Dicer Suppresses Hepatocellular Carcinoma via Interleukin-8 Pathway

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ABSTRACT

BACKGROUND: Elevated level of interleukin-8 (IL-8) promotes hepatocellular carcinoma (HCC) development and contributes to poor prognosis. Previously, we have proved that Dicer inhibits HCC progression. In this study, we evaluated the potential interaction between IL-8 and Dicer as well as their influence on HCC.

METHODS: Hepatocellular carcinoma cells of SMMC-7721 were divided into 2 groups for subsequent analysis: pCMV-Dicer group for Dicer-overexpressing lentivirus transfected cells (pCMV-Dicer cells) and pCMV-NC group for empty lentivirus transfected cells (pCMV-NC cells). Cell Counting kit-8 (CCK8), wound healing, and transwell were used to evaluate the inhibitory effect of Dicer overexpression on proliferation, migration, and invasion of HCC cells. The level of IL-8 was measured by flow cytometry bead-based immunoassays. Male nude BALB/c mice injected with pCMV-Dicer or pCMV-NC cell suspensions was used for transplant of HCC tumor.

RESULTS: We found that the secretion of IL-8 was reduced in the medium of pCMV-Dicer cells ($P = .027$). Recombinant human IL-8 (rhIL-8) reversed the inhibitory effect of Dicer on proliferation ($P < .01$), migration ($P = .003$), and invasion ($P = .001$), whereas IL-8 inhibitor of reparixin enhanced inhibitory effect of Dicer on proliferation ($P < .05$), migration ($P = .008$), and invasion ($P = .000$). Lenvatinib downregulated the IL-8 level of HCC cells ($P = .000$) as well as promote Dicer-induced inhibition for HCC cells referring to proliferation ($P < .05$), migration ($P = .000$), and invasion ($P = .000$). Animal experiments also demonstrated that Dicer cooperated with lenvatinib to inhibit the growth of HCC tumors ($P < .05$).

CONCLUSIONS: Dicer cooperated with lenvatinib to inhibit HCC growth via downregulating IL-8, and Dicer displayed its potential capability to enhance the anti-tumor effect of lenvatinib.

KEYWORDS: Dicer, interleukin-8, reparixin, lenvatinib, hepatocellular carcinoma

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Introduction

Primary liver cancer (PLC) is the seventh most common cancer and the second leading cause of cancer mortality worldwide, and approximately 75% of PLC is hepatocellular carcinoma (HCC).[1] In early stage of HCC, liver resection, liver transplant, and ablative techniques are efficient treatment methods.[2] However, about 40% of HCC patients are diagnosed at the advanced stage when surgery may not be effective, and the options for these patients include the administration of tyrosine kinase inhibitor (TKI), immune checkpoint inhibitors (ICIs), and anti-angiogenic drugs.[3,4] The TKI activity of lenvatinib showed noninferiority to sorafenib as the first-line treatment for HCC.[5,6] It can reduce the infiltration of regulatory T-cells (Tregs) and inhibit the transforming growth factor β (TGF-β) signaling pathway thereby improving tumor microenvironment.[7] The overall response rate of lenvatinib is only 24% with the mechanisms of drug resistance unknown.[8]

MicroRNAs (miRNAs) are small noncoding RNAs that have 18 to 25 nucleotides, binding to the 3′-untranslated target region to regulate cell proliferation, differentiation, and apoptosis, and influence tumor progression and metastasis.[9,10] Dicer is a cytoplasmic RNaseIII enzyme involved in cell proliferation and apoptosis by cleaving premicroRNAs into mature microRNAs and short interfering RNAs in the cytoplasm.[11] Low expression of Dicer is related to poor prognosis in cancer patients including breast cancer,[12] gastric cancer,[13] clear cell renal cell carcinoma,[14] and colorectal cancer.[15] We confirmed previously that Dicer-suppressed HCC by inhibiting the proliferation, invasion, and migration of HCC cells.[16] We also found that Dicer enhanced the bevacizumab-related inhibition of HCC by blocking vascular endothelial growth factor (VEGF) pathway; however, the underlying mechanism has not been clarified.[17]

Interleukin-8 (IL-8), also known as CXCL8, is an important chemokine of the CXC family responsible for the

recruitment and activation of neutrophils at inflammation sites. Not only malignant tumor cells but also myeloid cells and fibroblasts infiltrating tumors can produce IL-8.[18,19] C-X-C chemokine receptor 1 (CXCR1) and C-X-C chemokine receptor 2 (CXCR2) are the 2 specific receptors on the cell surface. IL-8 activates downstream signaling pathways by combining with CXCR1/2, participating in tumor cell proliferation, invasion, epithelial-mesenchymal transition (EMT), and angiogenesis.[20,21] Interleukin-8-CXCR1/2 remodels the tumor microenvironment by recruiting neutrophils and myeloid-derived suppressor cells (MDSCs).[22] Elevated IL-8 is associated with poor prognosis in a variety of tumors such as head and neck squamous cell carcinoma,[23] ovarian cancer,[24] esophageal squamous cell carcinoma,[25] and gastric cancer.[26] In HCC, the up-regulation of IL-8 expression is also related to the enhancement of tumor cell malignant behaviors, leading to poor prognosis.[27] It could enhance the invasiveness of liver cancer cells by activating PI3K/Akt pathway.[28] Interleukin-8-CXCR1 axis enhances endothelium permeability to promote tumor cells vascular metastasis by increasing Tregs in liver cancer microenvironment.[29] In this study, we evaluated the interaction of Dicer with IL-8 as well as their influence on HCC progression.

Materials and Methods

Cell culture and transfection

Human HCC cell line SMMC-7721 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY). The cells were incubated in a humidified incubator containing 5% CO_2 at 37°C. Recombinant human IL-8 (rhIL-8, 110719) was purchased from Peprotech, (Rocky Hill, NJ). Reparixin (CSN15950) was purchased from CSNpharm; (Chicago, IL) and lenvatinib (E7080 A2174) was obtained from APExBIO (Houston, TX).

SMMC-7721 cell transfected with green fluorescent protein (GFP)-tagged Dicer-overexpressed lentivirus (pCMV-Dicer) or negative control lentivirus (pCMV-NC)[17] was used for functional assay. The successful transfection of Dicer was confirmed by western blot analysis as described previously.[17]

Cell proliferation assay

Cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used for cell proliferation assay. Approximately 2×10^3 cells were seeded into 96-well plates with 100 μL medium per well and incubated at 37°C with 5% CO_2. After incubation with 10 μL CCK-8 for 2 h, the absorbance of each cell was calculated with a microplate reader (Bio Tek, Winooski, VT) at wavelength of 450 nm at different time points (0, 24, 48, 72, and 96 h).

Wound healing assay

Cells were seeded on 6-well plates and cultured in DMEM medium with 10% FBS. When the cells reached approximately 100% density, 2 straight scratches were drawn using a 10 μL pipette tip in each well, on the surface of the plates. After washing twice with phosphate-buffered saline (PBS), the cells were cultured further in a fresh medium with 2% FBS for 24 h. An inverted microscope (Nikon, Tokyo, Japan) was used for capturing images at 0 and 24 h. Cell migration rates were calculated as the width of the difference between 24 h and 0 h divided by the initial width.

Invasion assay

Cell invasion capacity was assayed using 24-well transwell chambers with 8 μm pore size (Corning, New York, NY) precoated with Matrigel (1:20, BD Biosciences, NJ). First, the cells were cultured in DMEM with 2% FBS before the experiment for 12 to 24 h. We seeded 4×10^4 or 6×10^4resuspended cells with 200 μL serum-free DMEM in the upper chamber, while 600 μL DMEM medium with 10% FBS was placed in the lower chamber. After incubation for 48 h at 37°C in 5% CO_2 atmosphere, the cells in the upper chamber were wiped gently. Later, the remaining cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 30 min. The stained cells were counted using an inverted microscope (Nikon, Tokyo, Japan) in 5 random fields (magnification 200×) for each membrane.

Cytokines detection

Interleukin-8 level was detected by flow fluorescence immunomicrobeads assay. Cells were cultured for 72 h in an incubator, and the supernatant was used for IL-8 measurement using LEGENDplex Multi-analyte flow assay kit (Biolegend, San Diego, CA) according to manufacturer's instructions as we have previously described.[30]

Animal experiment

All animal experiments were approved by the Animal Ethics Committee of the Fourth Hospital of Hebei Medical University on December 3, 2021 (ID: 2021239). Twenty 4-week-old athymic male nude BALB/c mice were purchased from Charles River Laboratories [Beijing, China; permission no. SCXK (Jing) 2016–0006]. The 5×10^6 SMMC-7721 pCMV-NC or pCMV-Dicer cells were resuspended in 200 μL serum-free DMEM and subcutaneously infected into the right shoulder of nude mice. After 12 days, the mice were randomly divided into 4 groups (n = 5): group pCMV-NC, group pCMV-Dicer, group pCMV-NC plus lenvatinib (lenvatinib 10 mg/kg), and group pCMV-Dicer plus lenvatinib (lenvatinib 10 mg/kg). As control, groups pCMV-NC and pCMV-Dicer were given 0.5%

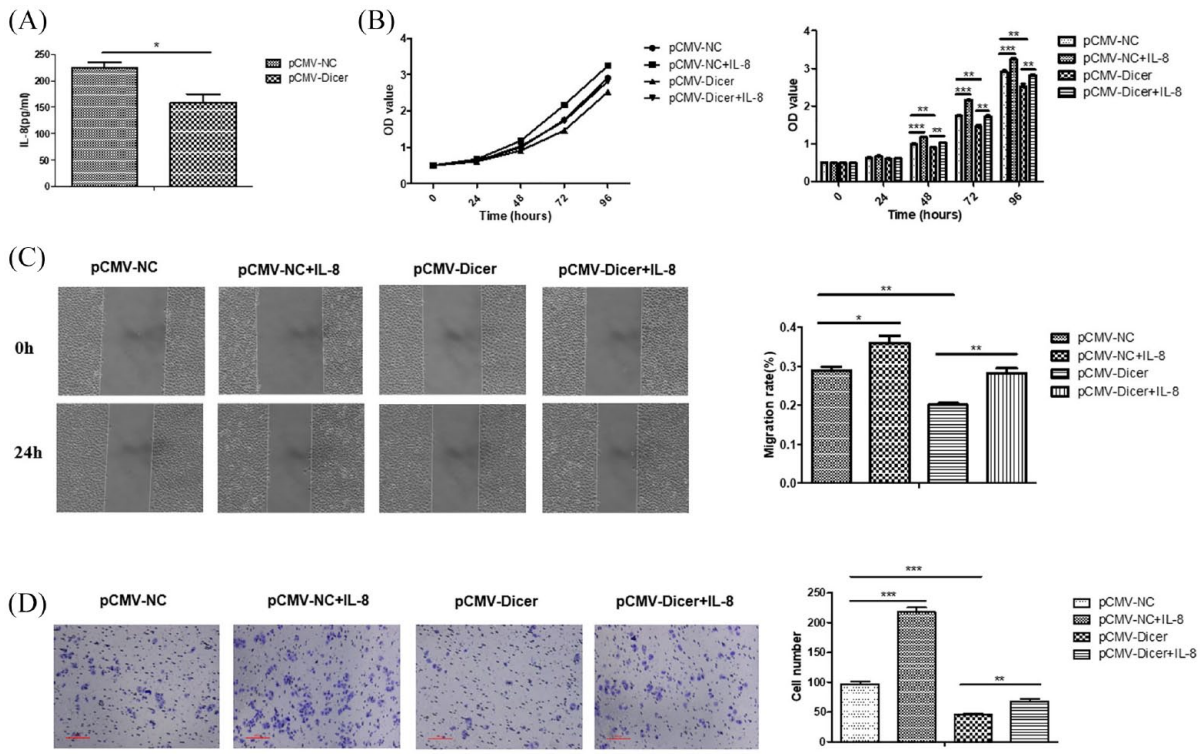

Figure 1. IL-8 offsets the suppression of Dicer on HCC cells: (A) Dicer downregulates the secretion of IL-8 in SMMC-7721 cells. The level of IL-8 detected by flow fluorescence immunomicrobeads assay (n = 3). (B) The cell proliferation analysis by CCK-8 assay. (C) The cell migration analysis by wound healing assay. (D) The cell invasion analysis by transwell assay with 4×10^4 cells in the upper chamber.
CCK-8 indicates cell counting kit-8; HCC, hepatocellular carcinoma; IL-8, interleukin-8; OD, optical density; pCMV-NC, SMMC-7721 cell transfected with pCMV lentivirus as negative control; pCMV-Dicer, SMMC-7721 cell transfected with Dicer-overexpressed pCMV lentivirus.
*$P < .05$.
**$P < .01$.
***$P < .001$.

methyl cellulose instead. All groups were given intragastric administration once every 2 days. Tumor sizes were measured every 4 days and calculated using the equation V (mm^3) = Length × (Width)^2/2. The mice were all sacrificed by cervical dislocation 16 days after the lenvatinib treatment, and the tumor tissues were stored for future use.

Statistical analysis

All statistical analyses were performed using SPSS statistics software, version 21.0 (Chicago, IL, USA). The measurement data were presented as mean ± standard deviation. The differences between the 2 groups were obtained by Student's *t*-test. $P < .05$ was considered to be statistically significant.

Results

Dicer suppresses HCC through IL-8

We overexpressed Dicer in HCC cell line of SMMC-7721 to evaluate the IL-8 level in the medium. As shown in Figure 1A, the secretion of IL-8 significantly decreased upon Dicer overexpression ($P = .027$), which indicated that Dicer inhibited IL-8 expression. The Dicer overexpression significantly inhibited the proliferation from 48 h to 96 h (Figure 1B, $P < .01$), migration (Figure 1C, $P = .001$) and invasion (Figure 1D, $P = .000$) of HCC cells when compared with those of pCMV-NC cells.

To evaluate whether Dicer inhibited HCC through IL-8 pathway, we incubated rhIL-8 with HCC cells at a concentration of 100 ng/mL for 96 h. RhIL-8 increased pCMV-NC cells proliferation from 48 to 96 h (Figure 1B, $P = .000$), migration (Figure 1C, $P = .026$) and invasion (Figure 1D, $P = .000$). It also neutralized Dicer-induced HCC cells inhibition on proliferation from 48 to 96 h (Figure 1B, $P < .01$), migration (Figure 1C, $P = .003$) and invasion (Figure 1D, $P = .001$) compared to pCMV-Dicer cells without rhIL-8. These data demonstrated that IL-8 could neutralize the Dicer-induced inhibition of HCC cells.

To further check whether the Dicer-modified HCC outcome through IL-8 pathway, the IL-8 receptor inhibitor of reparixin was used for subsequent analysis with 50 μg/mL work concentration. As shown in Figure 2, it inhibited cell proliferation from 72 to 96 h (Figure 2A, $P < .05$), migration (Figure 2B, $P = .002$) and invasion (Figure 2C, $P = .000$) after incubating pCMV-NC cells with reparixin. The same and more obvious trend was obtained for cell proliferation from 48 to 96 h (Figure 2A, $P < .05$), migration (Figure 2B, $P = .008$)

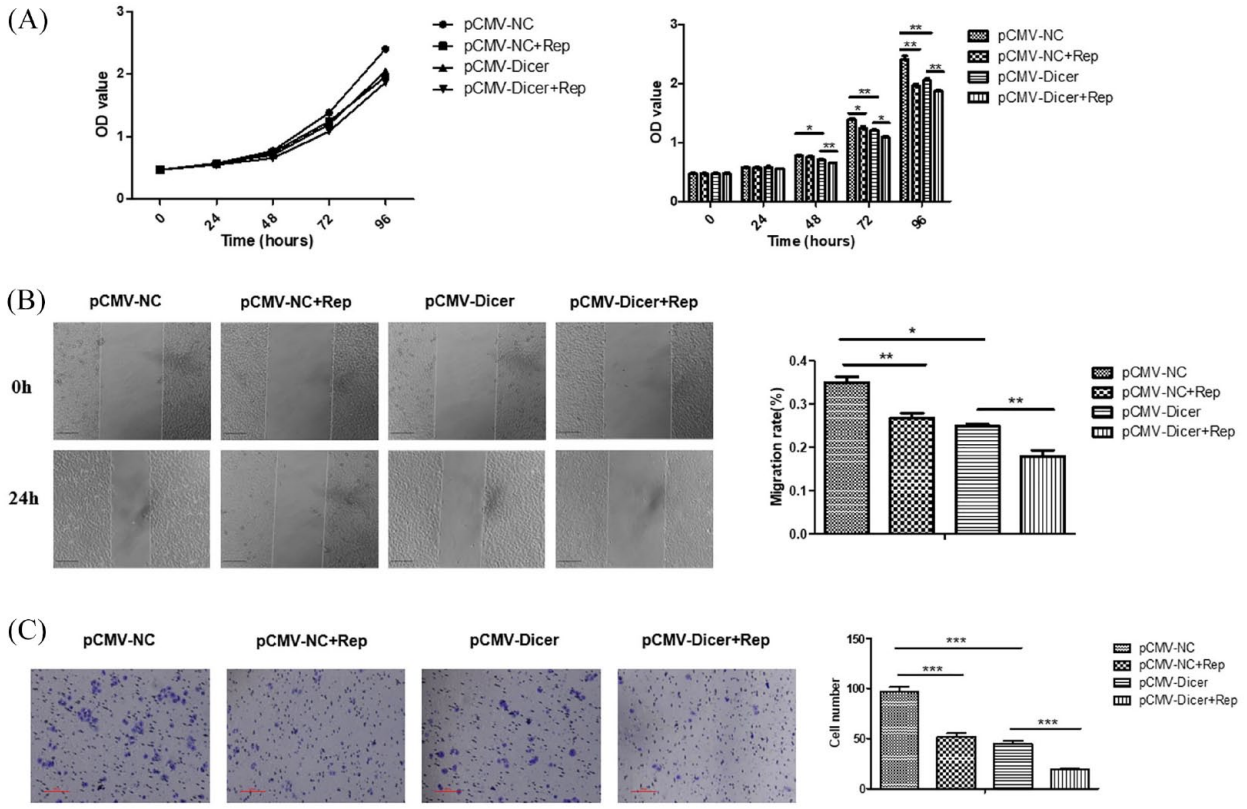

Figure 2. Reparixin enhances the suppression of Dicer on HCC cells: (A) The cell proliferation analysis by CCK-8 assay. (B) The cell migration analysis by wound healing assay. (C) The cell invasion analysis by transwell assay with 4×10^4 cells in the upper chamber.
CCK-8 indicates cell counting kit-8; HCC, hepatocellular carcinoma; Rep, reparixin; OD, optical density; pCMV-NC, SMMC-7721 cell transfected with pCMV lentivirus as negative control; pCMV-Dicer, SMMC-7721 cell transfected with Dicer-overexpressed pCMV lentivirus.
*$P < .05$.
**$P < .01$.
***$P < .001$.

and invasion (Figure 2C, $P = .000$) when incubated with pCMV-Dicer cells and reparixin. These data indicated that reparixin could enhance growth suppression induced by Dicer in HCC cells. Thus, Dicer inhibits HCC cells growth by regulating IL-8 pathway.

Lenvatinib cooperated with dicer to inhibit HCC

We incubated SMMC-7721 cells with lenvatinib (E7080) at 20 μM concentration for 72 h. The secretion of IL-8 decreased significantly after lenvatinib treatment (Figure 3A, $P = .000$). This result showed that lenvatinib inhibited the expression of IL-8. In addition, lenvatinib inhibited pCMV-NC cells proliferation from 48 to 96 h (Figure 3B, $P < .05$), migration (Figure 3C, $P = .000$), and invasion (Figure 3D, $P = .000$). When cocultured lenvatinib with pCMV-Dicer cells subsequently, it provided additive suppression with pCMV-Dicer cells on proliferation from 24 to 96 h (Figure 3B, $P < .05$), migration (Figure 3C, $P = .000$) and invasion (Figure 3D, $P = .000$). Next, we cocultured pCMV-NC or pCMV-Dicer cells with the same concentration of lenvatinib for 72 h. The results showed that the level of IL-8 in pCMV-Dicer cells was obviously decreased than that in pCMV-NC cells (Supplementary Figure S1, $P = .006$). These data demonstrated that Dicer combined with lenvatinib had a more obvious inhibitory effect on the expression of IL-8 compared with lenvatinib alone. So, we believed that lenvatinib enhanced Dicer-related suppression of HCC by decreasing IL-8 secretion *in vitro*.

The cooperation of Dicer and lenvatinib was evaluated *in vivo*. The growth of pCMV-Dicer xenograft decreased significantly compared to pCMV-NC xenograft from 12 to 16 days after implantation (Figure 4A and B, $P < .05$). In addition, lenvatinib provided additive suppression in both pCMV-NC group (Figure 4A and B, $P < .01$) from 8 to 16 days and pCMV-Dicer group (Figure 4A and B, $P < .05$) from 4 to 16 days. Furthermore, Dicer exhibited lenvatinib-related inhibition trend for HCC xenograft compared with pCMV-NC plus lenvatinib xenograft at a marginal statistical level in 16 days (Figure 4A and B, $P = .074$). These data suggested that lenvatinib could enhance the tumor inhibition of Dicer, and Dicer displayed its potential capability to enhance the antitumor effect of lenvatinib *in vitro* and *vivo*.

Discussion

Based on Dicer-induced growth inhibition on HCC cells *in vitro* and *in vivo*,[17,31] we found that Dicer could inhibit HCC

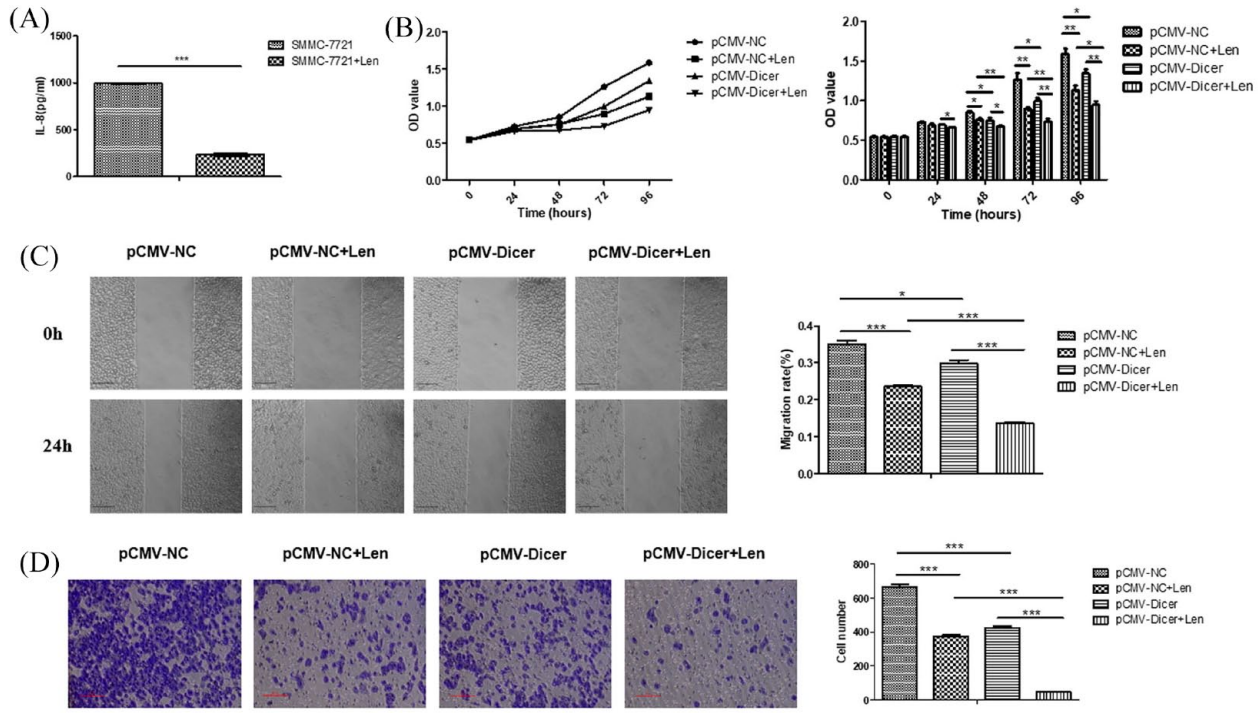

Figure 3. Lenvatinib has cooperative effect on the tumor inhibition of Dicer in vitro: (A) Lenvatinib downregulates the secretion of IL-8 in HCC cells (n = 3). (B) The cell proliferation analysis by CCK-8 assay. (C) The cell migration analysis by wound healing assay. (D) The cell invasion analysis by transwell assay with 6×10^4 cells in the upper chamber.

CCK-8 indicates cell counting kit-8; HCC, hepatocellular carcinoma; IL-8, interleukin-8; Len, lenvatinib; Rep, reparixin; OD, optical density; pCMV-NC, SMMC-7721 cell transfected with pCMV lentivirus as negative control; pCMV-Dicer, SMMC-7721 cell transfected with Dicer-overexpressed pCMV lentivirus.

*$P < .05$.

**$P < .01$.

***$P < .001$.

by downregulating IL-8. We also found that Dicer in cooperation with lenvatinib inhibited the growth of HCC via their common target of IL-8 pathway, which implied potential clinical application of Dicer expression on lenvatinib antitumor effect, but whether Dicer can overcome the lenvatinib resistance need to be furtherly evaluated. Elevated IL-8 in the tumor microenvironment plays a key role in the progression and metastasis of tumor.[32] Interleukin-8 introduces granulocytic MDSCs to form neutrophil extracellular traps (NETs), which promote metastasis and thrombus formation.[33,34] Moreover, IL-8-CXCR1/2 axis may regulate cancer stem cells (CSCs) proliferation and self-renewal to promote tumor progression and metastasis.[35] The Dicer-IL-8 pathway might modify HCC development through the above mechanisms.

We have proven that Dicer inhibited HCC via downregulation of VEGF.[17] Then, we suspected that Dicer might modify HCC outcome via IL-8-VEGF axis, but VEGF expression did not change upon IL-8 or reparixin incubation in HCC cells (data not shown). This has proved that a positive feedback loop exists between EMT and IL-8 and that IL-8 promotes the occurrence of EMT, whereas EMT induces the production of IL-8 in colon cancer correspondingly.[21,36,37] Dicer also modulates EMT via hypoxia-inducible factor 1α (HIF-1α) in HCC.[38] We compared the EMT markers upon IL-8 or raparixin incubation in pCMV-Dicer and pCMV-NC cells, and no expressional difference in these markers was found by western blot analysis (data not shown). Interleukin-8 expression was regulated by some miRNAs. The overexpression of miR-93 and miR-17 inhibited the production and release of IL-8; miR-140-3p inhibited only the expression of IL-8, while miR-155 promoted IL-8 production.[39-41] It is highly possible that the miRNA synthetase of Dicer might affect IL-8 secretion by regulating miRNA expression.

Interleukin-8 in peripheral blood and liver fibrotic tissue is associated with liver fibrosis.[42] It activates hepatic stellate cells (HSCs) and stimulates alpha-smooth muscle actin (α-SMA) expression for the formation and activation of stress fibers.[43] The biomarkers for liver fibrosis did not change significantly when we cocultured IL-8 with HCC cells for 72 h (data not shown). However, these findings could not exclude the possibility that Dicer-mediated downregulation of IL-8 reduced HSCs fibrosis thereby inhibiting HCC progress.

Conclusions

In conclusion, Dicer in cooperation with lenvatinib inhibits HCC growth via downregulating IL-8. Dicer displayed its potential capability to enhance the antitumor effect of lenvatinib.

Author Contributions

ZG designed the original experiments. ZG and JW directed the progress of the experiments. XH performed the

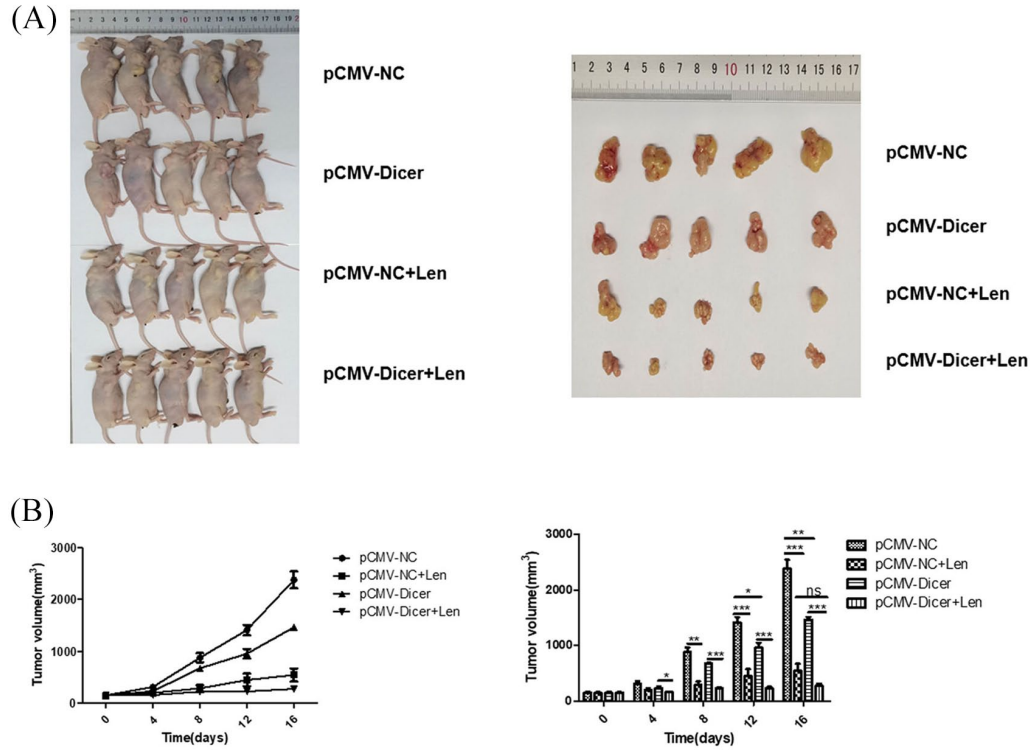

Figure 4. Lenvatinib has cooperative effect on the tumor inhibition of Dicer *in vivo* (n = 5): (A) 5×10^6 SMMC-7721 pCMV-NC or pCMV-Dicer transfected cells were infected into nude mice for 16 days. (B) Subcutaneous xenograft tumor volume was measured every 4 days.
Len indicates lenvatinib; OD, optical density; pCMV-NC, SMMC-7721 cell transfected with pCMV lentivirus as negative control; pCMV-Dicer, SMMC-7721 cell transfected with Dicer-overexpressed pCMV lentivirus.
*$P < .05$.
**$P < .01$.
***$P < .001$.

experiments. XH, ZS, RL, JS, LM, and FY interpreted the data and reviewed the manuscript. XH and ZG wrote the manuscript. All authors had final approval of the submitted and published versions.

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Supplemental Material

Supplemental material for this article is available online.

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